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13. ABSTRACT (Maximum 200 Words)

We studied the role and regulation of a secreted, FGF binding protein (FGF-BP) in breast cancer.
We established a method to detect FGF-BP mRNA in paraffin-embedded archival breast cancer samples using in situ hybridization. We show feasibility of the technique for breast cancer tissues.

We surveyed breast cancer cell lines and show that EGF-receptor overexpressing MDA-MB 468 human breast cancer cells express high levels of FGF-BP mRNA that is detectable by Northern blot analysis.
We demonstrate that the FGF-BP gene is regulated in these breast cancer cells by the growth factor EGF.
We show that the MAP kinase p38 is crucial in the transduction of the EGF signal towards FGF-BP regulation in MDA-MB 468 cells.

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INTRODUCTION

Fibroblast growth factors (FGFs) and a potential function for a FGF-binding protein (BP)

It is only partly understood how FGFs become solubilized from their extracellular storage and thus activated in embryonic or in tumor tissues that require angiogenesis for their growth. One established mechanism that can solubilize FGF-2 from this storage is via non-covalent binding to a secreted carrier protein. Such a secreted binding protein for FGFs was described by D. Sato's laboratory in 1991 [1]. We hypothesized that this BP could be an important regulator that releases immobilized FGFs from their matrix storage site and thus activates them in vivo. Figure 1 illustrates the proposed mechanism of action of BP that will be elucidated in more detail further below.
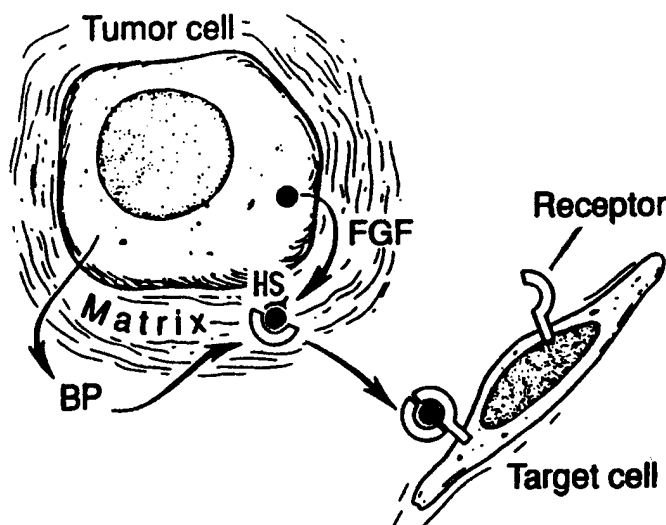

Figure 1. Model explaining the function of FGF-BP (BP) for the release of FGFs that are immobilized in the extracellular matrix. The secreted protein BP combined with high affinity into the immobilized FGS and release it in an active form.

The major focus of this cycle is to explore the expression and basis for the activation of FGF-BP expression in breast cancer.

Technial objectives this cycle:
Goal (1): To assess the significance of FGF-BP expression for the clinical course of breast cancer using archival samples with known outcome and defined pathological features. Task during Months 1 – 24.

Goal (2): To study which regulators of expression of the endogenous FGF-BP gene in FGF-BP-positive breast cancer cell lines. Task during Months 1 – 12.

BODY

TASK 1:
To assess the significance of FGF-BP expression for the clinical course of breast cancer using archival samples with known outcome and defined pathological features.

We had found expression of FGF-BP in a small series of breast cancer samples from our Cancer Center's tissue bank and from this proposed to study a larger set including non-malignant and premalignant lesions and compare this result to known pathological, molecular and clinical parameters.

**TASK 1; Work accomplished during the first award cycle (Months 1-12):**

Enhancing the method of detection of FGF-BP in the tumor cell compartment.

FGF-BP is a secreted protein that is released to the extracellular compartment (see Figure 1). Thus, detection of the protein in a given tissue does not reveal the cells producing the protein. This problem can be solved by detecting FGF-BP mRNA by in situ hybridization using tissue sections. We had already established in situ hybridization for FGF-BP mRNA in some tissues (frozen specimen, SCC, squamous cell carcinoma mostly). We first attempted to apply the method of detection to human breast cancer samples from the tissue archive. These are paraffin-embedded samples. Figure 1a shows an example of non-cancerous breast tissue and we found that this tissue rarely stains for FGF-BP mRNA and protein. Invasive carcinoma were found to stain positively for FGF-BP mRNA and protein in approximately 1/2 of the cases. Figure 1b shows an FGF-BP mRNA negative case and Figure 1c shows an FGF-BP mRNA positive case. The staining for mRNA is found in the cytosol of the positive cells - as expected - and the nuclei do not show a hybridization signal ("empty nuclei"). This is an important control to demonstrate a lack of non-specific cross-hybridization with DNA.

FGF-BP - - +

a *b* *c*
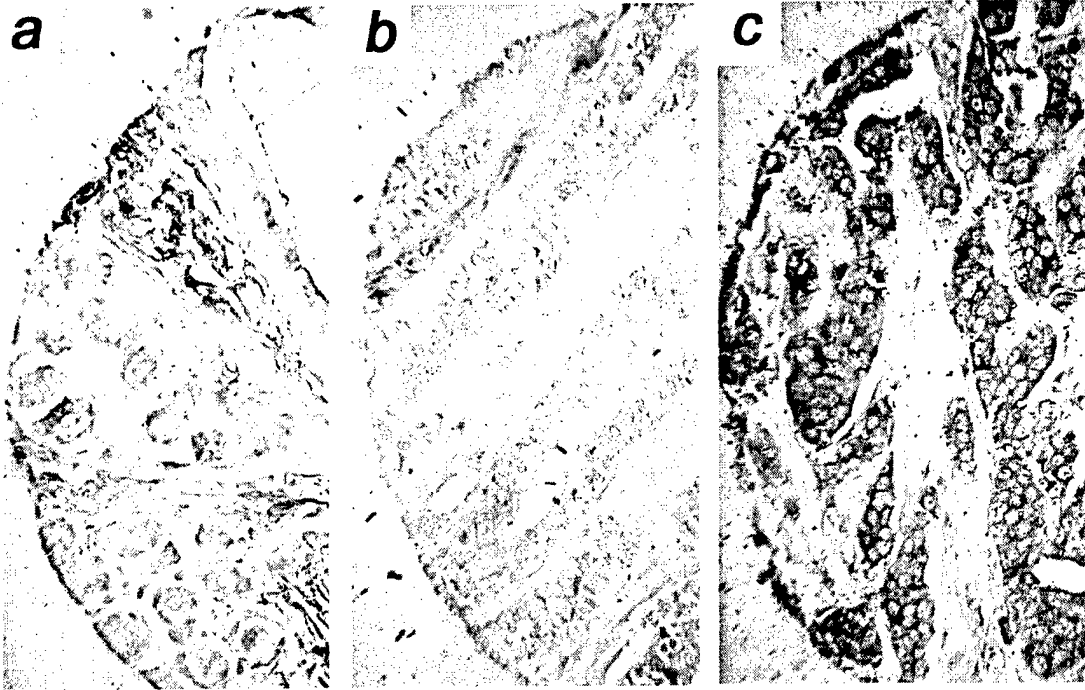
normal breast *invasive breast cancer*

Figure 1. Expression of FGF-BP in human breast tissues.
In situ hybridization for FGF-BP mRNA using a tissue microarray (TMA) with paraffin-embedded normal breast (a) and invasive breast cancer (b,c) tissues. Normal breast and one of the breast cancer samples (b) were scored as negative for FGF-BP whereas the second breast cancer sample shown (c) was scored as positive.

Note: The FGF-BP mRNA expression is confined to the epithelial tissues. The nuclei of the positive cells do not stain for the mRNA.
(These samples were from a TMA obtained through the NIH Cooperative Breast Cancer Tissue Resource CBCTR. The samples shown were present on the same slide and were thus all hybridized at the same time.)

Methods:

In situ Hybridization
The expression of FGF-BP mRNA in human breast tissue samples was assessed by in situ hybridization. The FGF-BP riboprobe consisted of a 668 bp internal sequence of FGF-BP cDNA [2], sub-cloned into the pRc/CMV vector (5.5 kb, Invitrogen). Digoxigenin-labeled antisense and sense riboprobes were made using the DIG RNA labeling kit

(Roche) according to protocol. Tissue sections were cut (4 micrometers) and mounted on (+)-charged glass slides (Fisher Scientific; Pittsburgh, PA) using standard histology technique. In addition, tissue microarrays (TMAs) from the NCI Cooperative Breast Cancer Tissue Resource (CBCTR) were used. This TMA contained samples from invasive cancer as well as in situ carcinoma and control breast tissues from reduction mammoplasty.

TASK 1; INTERPRETATION OF THE DATA.

In situ hybridization for FGF-BP is a selective method to detect the mRNA in paraffin-embedded, archival samples. In addition to the above breast tissues, other tissues were also stained with the same approach and showed a selective expression of FGF-BP e.g. in colon cancer versus normal colon archival tissues.

TASK 2:
To study regulators of the endogenous FGF-BP gene in breast cancer cell lines.

Work accomplished during the first award cycle (Months 1 – 12):

MDA-MB468 human breast cancer cells as a model to study regulation of FGF-BP.

To find a tissue culture model that recapitulated the high FGF-BP expression that we observed in our preliminary studies with invasive breast cancer samples, we screened a number of cell lines for FGF-BP mRNA expression. We assayed FGF-BP mRNA expression, rather than protein expression, because FGF-BP is a secreted protein [3], and therefore is difficult to measure using whole cell extracts. We reported previously that most breast cancer cell lines (including MCF-7 cells) are negative for FGF-BP expression by Northern analysis [3], and we now found that MDA-MB-468 cells have high FGF-BP expression (Figure 1). Interestingly, MDA-MB-468 is a breast cancer cell line that can be invasive and metastatic *in vivo* when injected into mice along with angiogenesis-stimulating Matrigel [4]. In addition, MDA-MB-468 cells have been characterized as showing high expression of the EGF receptor [5] and are EGF responsive for invasion [6]. Overexpression of the EGF receptor is a poor prognostic indicator in breast cancer [7] presumably through EGF stimulation of a more invasive phenotype although the precise target genes are not yet defined.

FGF-BP expression MCF-7 cells was not detectable even when cells grown in full serum that contains estradiol and growth factors. However, one of the drug-resistant MCF-7 derivative cell lines, MCF-7/Adr, expresses detectable levels of FGF-BP by Northern blot (Fig. 1). This cell line has lost estrogen responsiveness and has a changed genotype relative to the original estrogen-responsive MCF-7 cell line.
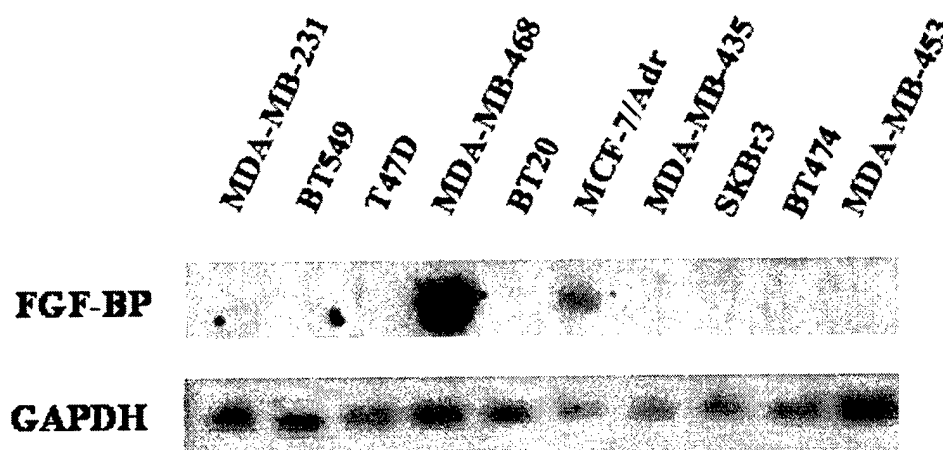

Figure 1. Northern blot analysis.
FGF-BP mRNA and a control (GAPDH mRNA) in human breast cancer cell lines is shown. Details at the end of this section under "Methods".

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EGF effect on FGF-BP expression in MDA-MB-468 human breast cancer cells.
Treatment of MDA-MB-468 cells with 10 ng/ml EGF resulted in a rapid increase in the steady-state levels of FGF-BP mRNA (Figure 2A). Induction of FGF-BP mRNA was observed after 1 hour of treatment and was maximal after 6 hours with an average increase of 2.5- to 3-fold (Figure 2B).

To discern between the possible signaling pathways involved in EGF induction of FGF-BP in MDA-MB-468 cells, we tested pharmacological inhibitors of signal transduction molecules at various concentrations for their effect on FGF-BP regulation. Treatment with the EGFR tyrosine kinase inhibitor PD153035 resulted in a significant concentration dependent inhibition of EGF induction of FGF-BP mRNA (Figure 2C). In addition to EGFR tyrosine kinase activity, protein kinase C is also involved in the EGF effect since the bisindoylmaleimide PKC inhibitors Ro 31-8220 [8], at concentrations of 1 mM and 10 mM, and calphostin C [9] were both able to significantly inhibit the EGF induction of FGF-BP (Figure 2C).

To determine the role of the further downstream signaling pathways on EGF induction of FGF-BP, we utilized MAPK kinase (MEK1/2) and p38 MAP kinase inhibitors [10,11]. The MEK1/2 specific inhibitor U0126 was only effective at higher concentrations of 10 µM and 20 µM, and not at the concentration of 1 mM (Figure 2C); 1µM of U0126 is usually used to specifically inhibit MEK-induced signaling [12]. In contrast, treatment with increasing concentrations of the p38 MAPK inhibitor SB202190 resulted in a concentration-dependent inhibition of EGF-induced FGF-BP mRNA (Figure 2C).
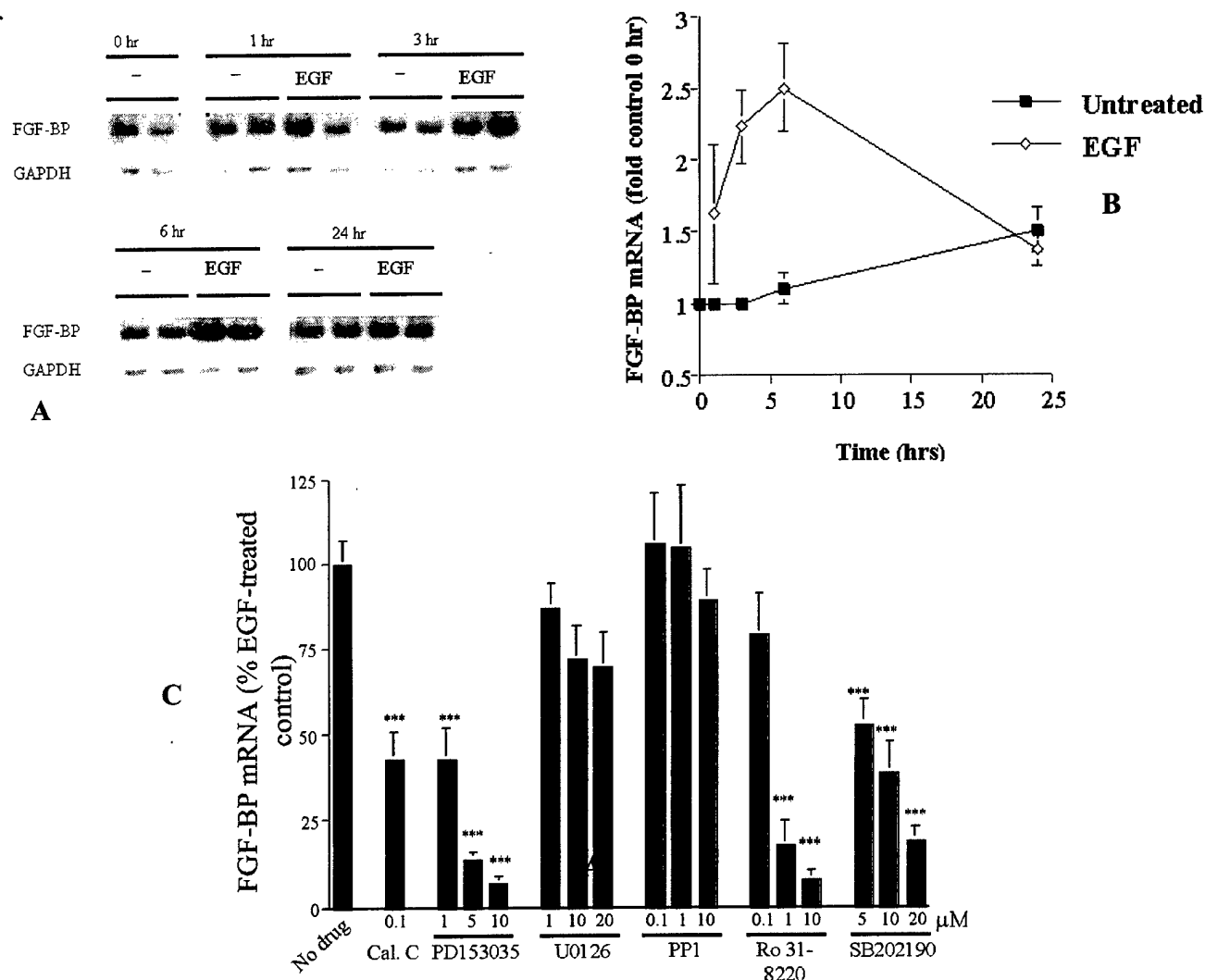
Figure 2 is on the next page !

Figure 2. **Induction of FGF-BP mRNA by EGF in MDA-MB-468 cells.** *A,* A representative Northern blot analysis showing FGF-BP mRNA and a control (GAPDH mRNA) done in duplicate. MDA-MB-468 cells were treated with or without 10 ng/ml EGF for the indicated times. *B,C,* Results from quantation of Northern blots. Signal intensities were quantified by PhosphorImager and normalized to the control mRNA, GAPDH. *B, Closed squares,* represent control (untreated) levels, and open squares represent EGF treatment mRNA levels expressed as fold over control-0 hrs. Values represent the mean and S.D. of at least two separate experiments. *C,* FGF-BP mRNA levels from MDA-MB-468 cells treated with 10 ng/ml EGF for 6 hours. Cells were pretreated for 1 hour with vehicle alone or at the indicated concentrations of: Calphostin C (PKC inhibitor), PD153035 (EGFR tyrosine kinase inhibitor), U0126 (MEK1/2 inhibitor), PP1 (c-Src inhibitor), Ro 31-8220 (PKC inhibitor), SB202190 (p38 MAPK inhibitor). Values are expressed relative to mRNA levels after EGF treatment alone (without inhibitor), which was set to 100%. Basal FGF-BP level (without EGF or inhibitor) was approximately 30%. Values represent mean and S.E. from of at least three separate experiments. Statistically significant differences relative control conditions (EGF only) (***, p<0.0001, t-test or ANOVA; Prism/Graphpad Program).

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TASK 2; Methods:

Cell Culture and Reagents
The MDA-MB-468 human breast cancer cell line, and the ME-180 human cervical squamous cell carcinoma cell line were obtained from American Type Culture Collection (ATCC; Manassas, VA). Cells were cultured in improved minimum essential medium (IMEM) with 10% fetal bovine serum (Invitrogen Inc.; Carlsbad, CA). Human recombinant EGF was purchased from Collaborative Biochemical Products (Bedford, MA). Tyrphostin AG1517 (PD153035), Ro 31-8220 (bisindoylmaleimide IX) and PP1 were purchased from Alexis Corp. SB202190 was purchased from Calbiochem (San Diego, CA). U0126 was purchased from Promega. Calphostin C was purchased Sigma-RBI (Natick, MA). Wortmannin was purchased from Biomol (Plymouth Meeting, PA). All compounds were dissolved in Me_2SO.

Northern Analysis-
MDA-MB-468 cells were grown to 80% confluence in 10-cm dishes, warmwashed twice in serum-free IMEM, and incubated for 16 hours in serum-free IMEM prior to treatment. Cells were pretreated for 1 hour with the indicated drug or with vehicle alone (Me_2SO; final concentration of 0.1%). EGF or anisomycin treatment was for 6 hours unless otherwise indicated. Total RNA was isolated with RNA STAT-60™ (Tel-Test Inc.; Friendswood, TX), and Northern analysis was carried out as described previously [13] using 20 mg of total RNA. Hybridization probes were prepared by random-primed DNA labeling (Amersham Biosciences; Piscataway, NJ) of purified insert fragments from human FGF-BP [3], and human GAPDH (Clontech; Palo Alto, CA). Quantification of mRNA levels was performed using a PhosphorImager (Amersham Biosciences).

TASK 2; INTERPRETATION OF THE DATA
These data suggest that p38 MAPK plays a dominant role in the induction of FGF-BP by EGF in MDA-MB-468 cells. Other intracellular targets for EGF receptor-induced intracellular signaling also potentially include members of the c-Src protein tyrosine kinase family. However, the c-Src family specific inhibitor PP1 [14] resulted only in a maximal 10% reduction in EGF-induced FGF-BP mRNA levels, only at the highest concentration used (10 µM (Figure 2C)). These data indicate only a minimal role for Src in EGF control of FGF-BP expression. The PI 3-kinase pathway also is not involved since treatment of MDA-MB-468 cells with 1 µM wortmannin did not have any significant effect on EGF induction of FGF-BP (data not shown). Overall, our pharmacological inhibitor data indicate major role for PKC, and more significantly, p38 MAPK in EGF effects on FGF-BP in breast cancer cells.

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KEY RESEARCH ACCOMPLISHMENTS

1. Established a method to detect expression of FGF-BP mRNA in paraffin-embedded, archival samples of invasive breast cancer.
2. Established a model breast cancer cell line (MDA-MB468) in which FGF-BP is expressed.
3. Demonstrated that FGF-BP in MDA-MB468 breast cancer cells is regulatable by the growth factor EGF.
4. Analysis of the pathway of EGF regulation of FGF-BP in these breast cancer cells.

REPORTABLE OUTCOMES

Publications (attached):

Tassi et al. (2001) J Biol Chem

CONCLUSIONS

- FGF-BP mRNA is detectable in invasive breast cancer samples.
- The FGF-BP gene is regulated in breast cancer cells by EGF.

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APPENDICES

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Enhancement of Fibroblast Growth Factor (FGF) Activity by an FGF-binding Protein*

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Fibroblast growth factor-binding protein (FGF-BP) 1 is a secreted protein that can bind fibroblast growth factors (FGFs) 1 and 2. These FGFs are typically stored on heparan sulfate proteoglycans in the extracellular matrix in an inactive form, and it has been proposed that FGF-BP1 functions as a chaperone molecule that can mobilize locally stored FGF and present the growth factor to its tyrosine kinase receptor. FGF-BP1 is upregulated in squamous cell, colon, and breast cancers and can act as an angiogenic switch during malignant progression of epithelial cells. For the present studies, we focused on FGF-1 and -2 and investigated interactions with recombinant human FGF-BP1 protein as well as effects on signal transduction, cell proliferation, and angiogenesis. We show that recombinant FGF-BP1 specifically binds FGF-2 and that this binding is inhibited by FGF-1, heparan sulfate, and heparinoids. Furthermore, FGF-BP1 enhances FGF-1- and FGF-2-dependent proliferation of NIH-3T3 fibroblasts and FGF-2-induced extracellular signal-regulated kinase 2 phosphorylation. Finally, in the chicken chorioallantoic membrane angiogenesis assay, FGF-BP1 synergizes with exogenously added FGF-2. We conclude that FGF-BP1 binds directly to FGF-1 and FGF-2 and positively modulates the biological activities of these growth factors.

Fibroblast growth factors (FGFs)[1] represent a family of over 20 distinct proteins that are widely expressed in various tissues. FGFs have been reported to be involved in both development and

adult tissue homeostasis, as well as in angiogenesis and cancer progression. FGF-2 (basic FGF), a 16–18-kDa protein, is one of the best-studied members of this family and has been shown to have a variety of biological effects in different cells and organ systems, including embryonic development, tumorigenesis, and angiogenesis (for a review, see Refs. 1 and 2).

FGF-2 interacts with low affinity cell surface and extracellular matrix heparan sulfate proteoglycans, which enable the growth factor to bind and activate its high affinity tyrosine kinase receptors (FGFRs), thereby forming a trimolecular active complex (3–6). It has been reported that cell surface heparan sulfate proteoglycans can modulate the action of FGF-2 by increasing its affinity for FGFRs (7). Moreover, heparan sulfate proteoglycans seem to protect FGF-2 from degradation by proteases in the extracellular environment (8, 9) and modulate the bioavailability of FGF-2, generating a local reservoir for the growth factor (10). The binding of FGF-2 to the cell surface receptor induces receptor tyrosine kinase dimerization and autophosphorylation (11). The phosphorylated FGFRs associate and subsequently activate SH2 domain-containing downstream signaling molecules, such as phospholipase Cγ (12, 13) and Src (14, 15). Moreover, upon ligand-dependent receptor autophosphorylation, adaptor proteins, such as Grb2 and Shc, link the FGFRs to the Ras/MAPK signaling cascade (16–18). Grb2 and Shc form a complex with the GDP/GTP exchange factor Son of Sevenless (Sos), which results in the translocation of Ras to the plasma membrane and its further activation by the exchange of GDP for GTP by Sos. Thus, activated Ras leads to the consecutive activation of a cascade of protein kinases involving Raf, MAPK/extracellular signal-regulated kinase kinase, and p42/44^{MAPK}, also known as extracellular signal-regulated kinase (ERK) 1 and 2 (16, 18).

FGF-2 lacks the classic leader sequence, which targets intracellular proteins for secretion to the extracellular environment, and several reports indicate that FGF-2 secretion occurs via endoplasmic reticulum- and Golgi-independent passive processes (19–21). In addition to the requirement for extracellular secretion, FGF-2 needs to be released and solubilized from the extracellular matrix (ECM) to act on its receptor. In comparison to other members of the FGF family, FGF-2 is tightly bound to the ECM and is a relatively abundant protein in numerous adult tissues, from which it can be extracted as a biologically active growth factor (22). In addition to FGF-2, several other less abundant members of this growth factor family are also stored in the ECM, although they have a lower affinity for glycosaminoglycans and are released more easily. Two distinct mechanisms by which locally stored FGF-2 can be released from the ECM have been described. One mechanism involves digestion of the sugar backbone in heparan sulfate proteoglycans by heparinases or other glycosaminoglycan-de-

grading enzymes (23–25). Binding of FGF-2 to an extracellular chaperone protein represents a separate mechanism for FGF-2 release and solubilization from the ECM. Studies from our laboratory have previously shown that the binding of FGF-2 to a secreted binding protein (FGF-BP) might represent such a mechanism (26, 27). Wu *et al.* (28) initially described FGF-BP as a low affinity heparin-binding protein isolated from human epidermoid carcinoma A431 cells. FGF-BP has been shown to bind to FGF-1 and -2 in a noncovalent, reversible manner. Moreover, FGF-BP protects and presents FGF-2 to its high affinity cell surface receptor (26, 27, 29), and a recent study (30) demonstrates the interaction of FGF-BP with perlecan, a heparan sulfate proteoglycan in the basement membrane. This most likely represents a local reservoir for FGF-BP. A related protein designated FGF-BP2 has recently been identified by our laboratory (2), and we will thus refer to the original protein as FGF-BP1.

FGF-BP1 is expressed below the level of detection by Northern blotting in normal adult human tissues, whereas its expression is significantly elevated in various tumors, including head and neck, skin, cervical, and lung squamous cell carcinomas (26, 30). In addition, FGF-BP1 is up-regulated in some colon cancers and breast adenocarcinomas (27). Furthermore, we have recently shown that phorbol esters as well as epidermal growth factor can up-regulate FGF-BP1 gene transcription (31–33). We reported previously (26) that expression of human FGF-BP1 cDNA in the FGF-2-positive SW-13 cells led these cells to grow anchorage independently. Likewise, whereas wild type SW-13 cells did not form tumors in nude mice, FGF-BP1-overexpressing SW-13 cells grew into highly vascularized tumors. Finally, we showed previously that the depletion of FGF-BP1 from squamous cell carcinoma and colon adenocarcinoma cell lines by ribozyme targeting resulted in a significant reduction of tumor growth and angiogenesis. In summary, these regulation, expression, and depletion experiments support a role for FGF-BP1 as a proangiogenic molecule in human tumors (27).

In the present study, we used recombinant FGF-BP1 protein to directly evaluate its binding to FGF-1 and -2 *in vitro* and study the functions of the protein. We found that FGF-BP1 was able to bind ^{125}I-FGF-2 in a dose-dependent and specific manner and can be competed by FGF-1 and FGF-2 as well as by different heparinoids. Furthermore, we studied the role of FGF-BP1 on the activation of the Ras/MAPK signaling pathway and on the mitogenic response of FGF-1- and FGF-2-treated NIH-3T3 fibroblasts. We demonstrate that FGF-2-induced ERK2 phosphorylation and proliferation were enhanced by the addition of FGF-BP1. Finally, in chorioallantoic membrane (CAM) assays, we found a significant FGF-BP1-dependent increase of FGF-2-mediated angiogenesis. Thus, our results indicate that the FGF-BP1 protein positively modulates the biochemical and biological activity of FGF-2 in multiple models.

MATERIALS AND METHODS

Cell Cultures—NIH-3T3 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc.) supplemented with 10% (v/v) calf serum. Sf-9 cells (BD PharMingen, San Diego, CA) were cultured in EX-Cell 400 media (JRH Bioscience, Lenexa, KS) supplemented with 5% (v/v) fetal calf serum in a humidified incubator at 27 °C in the absence of CO_2.

Recombinant Histidine-tagged FGF-BP1 Protein Purification—The His-tagged FGF-BP1 protein was produced by infecting Sf-9 cells with a baculovirus vector that contains an expression cassette for human FGF-BP1 (BAC-TO-BAC Baculovirus Expression System; Life Technologies Inc.). The baculovirus construct contains nucleotides 197–799 of the human FGF-BP1 cDNA, flanked bilaterally by cDNAs encoding six histidine residues. The FGF-BP1 cDNA fragment was inserted into pFASTBAC HTb donor plasmid, which was then transformed into bac-

mid-containing DH10BAC competent cells. LacZ-negative clones containing the recombinant bacmid were identified. The bacmid DNA was isolated and then transfected into Sf-9 cells to generate baculovirus. Infected Sf-9 cells were grown for 5 days, pelleted, and lysed in a buffer containing 6 M guanidine-HCl, 0.01 M Tris-HCl, and 0.1 M sodium phosphate, pH 8.0. Cell lysates were homogenized and then incubated for 1 h on ice. Cellular debris was removed by centrifugation at 10,000 × *g* for 15 min. Supernatant was loaded onto a Ni-NTA-Sepharose column (Qiagen, Hilden, Germany). The column was sequentially washed with buffers containing 30 mM sodium citrate, 300 mM NaCl, and decreasing pH values of 8, 6.3, 5.9, and 5.7, respectively. His-tagged FGF-BP1 was then eluted with three aliquots of 0.5 ml of the buffer at pH 4.5. Eluates were neutralized immediately and stored at 4 °C.

Silver Staining and Western Blot Analysis—30 μl of recombinant FGF-BP1 were resuspended with 5× Laemmli's buffer, boiled at 95 °C for 5 min, and separated by electrophoresis on a 4–20% gradient polyacrylamide gel. The protein was then detected by silver staining and after immunoblotting. Silver staining was performed as suggested by the manufacturer (Bio-Rad). The His-tagged protein was detected with a rabbit polyclonal anti-FGF-BP1 (27) or a mouse monoclonal anti-His antibody (Invitrogen Corp., Carlsbad, CA) that was then visualized by enhanced chemiluminescence detection using horseradish peroxidase-linked donkey anti-rabbit or anti-mouse immunoglobulin G as the secondary antibodies, respectively (Amersham Pharmacia Biotech).

Phosphorylation Studies—50% confluent NIH-3T3 cells were serum-deprived overnight and treated for 5 min with 2 and 10 ng/ml FGF-2 (Invitrogen Corp.) and recombinant FGF-BP1. Controls were left untreated. Cells were then washed with cold phosphate-buffered saline, pH 7.4, and subsequently lysed at 4 °C in a buffer containing 50 mM Tris-HCl, pH 8, 150 mM NaCl, 40 mM β-glycerophosphate, 1 mM EGTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, 50 mM sodium fluoride, 20 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 1 μg/ml pepstatin, and 100 μg/ml pefabloc. Cellular debris was removed by centrifugation at 14,000 rpm for 15 min. Phosphorylated proteins were immunoprecipitated from the cleared lysates by incubation with agarose-conjugated anti-phosphotyrosine (4G10; Upstate Biotechnology, Lake Placid, NY) monoclonal antibody for 2 h at 4 °C. Immunocomplexes were recovered by centrifugation and washed five times with cold lysis buffer. Samples were then resuspended with 15 μl of 2× Laemmli's buffer and boiled for 5 min at 95 °C. Alternatively, 10–50 μg of total protein cell extracts were resuspended with 5× Laemmli's buffer and heated for 5 min at 95 °C. Both immunoprecipitates and total cell lysates were separated on 10% SDS-polyacrylamide gels, transferred onto polyvinylidene difluoride membranes, and analyzed by immunoblot analysis. Tyrosine-phosphorylated proteins and ERK2 were detected with the corresponding mouse monoclonal antibody and then visualized by enhanced chemiluminescence detection using horseradish peroxidase-linked goat anti-mouse antibody (Amersham Pharmacia Biotech), respectively. Monoclonal anti-pan ERK antibody was purchased from BD Transduction Laboratories (Lexington, KY).

FGF/FGF-BP1 Binding Assays—100 ng/ml His-BP1 (a bilaterally hexahistidine-tagged FGF-BP1 protein) diluted in Tris-buffered saline (TBS) was incubated overnight in 96-well plates (EIA/RIA Strip Plate; Corning Inc., Corning, NY) at 4 °C with constant rocking. Excess unbound His-BP1 was removed by washing the wells twice with TBS. Nonspecific binding was blocked by the addition of 300 μl of LB medium (Bio 101, Carlsbad, CA) to the wells for 1 h at room temperature. Wells were then washed five times with TBS. ^{125}I-FGF-2 (1–20 ng/ml) was added to the wells and incubated for 2 h at room temperature with constant rocking. Unbound ^{125}I-FGF-2 was removed by washing the wells five times with TBS containing 2% Tween 20. In the competition assays, different amounts of FGF-1, FGF-2, His-BP1, pentosanpolysulfate (bene Chemie, Munich, Germany), and heparin or heparan sulfate (Sigma) were simultaneously added with ^{125}I-FGF-2. Binding of radiolabeled FGF-2 to His-BP1 was measured by counting the radioactive emission from the individual wells. ^{125}I-FGF-2 was purchased from Amersham Pharmacia Biotech. Human recombinant FGF-1 and FGF-2 were purchased from Life Technologies, Inc.

Protein-protein Interaction Studies on Protein Arrays (Protein Chip Assay)—The analysis was performed with a surface-enhanced laser desorption/ionization (34) (Protein Biology System I; Ciphergen, Palo Alto, CA). The different protein-containing preparations (1 μl of a 20 mg/ml solution) were placed on a normal-phase protein array, which was then washed, and 1 μl of a-cyano-4-hydroxy cinnamic acid (2 mg/ml) in 50% (v/v) acetonitrile and 0.5% (w/v) trifluoroacetic acid was added to the spot. The retained proteins were then subjected to mass spectrometry. For the analysis of the interaction of FGF-BP1 with
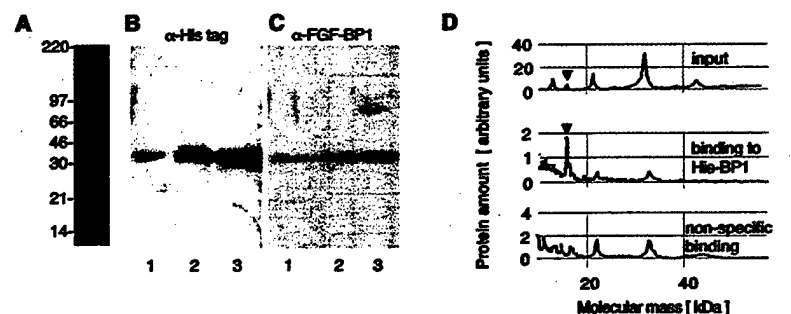
FIG. 1. **Silver stain, Western blots, and binding of FGF-2 to the recombinant His-BP1 protein.** *A*, silver stain of 30 μl of a pooled, chelate affinity-purified His-BP1 preparation separated by a 4–20% gradient SDS-PAGE. *B* and *C*, Western blots of 30-μl aliquots from consecutive affinity chromatography fractions loaded onto 4–20% SDS-PAGE. A blot with anti-His tag (*B*) and anti-BP1 (*C*) antibodies is shown. *D*, protein chip analysis using surface-enhanced laser desorption/ionization to assess FGF-2 binding to immobilized His-BP1. Mass spectrometry analysis of proteins is shown. *1*, input FGF-2 ligand preparation (FGF-2 spiked into cell growth media with 10% fetal calf serum). *2* and *3*, proteins present in the input preparation that bound to immobilized His-BP1 (*2*) or to background (*3*), respectively. The *arrowheads* indicate the peak corresponding to the FGF-2 protein used as a ligand.

FGF-2, 3 μl of a 240 mg/ml solution of FGF-BP1 in phosphate-buffered saline were applied to a preactivated protein array, which was then incubated overnight in a humidified chamber at 4 °C. The protein solution was removed, 3 ml of 1 M ethanolamine (pH 8.2) were added to each spot, and the array was incubated for an additional 30 min at room temperature. For further details, see Ref. 35.

Proliferation Assay—5 × 10³ NIH-3T3 fibroblasts were seeded in three replicates in 96-well plates for 8 h. Cells were serum-deprived for 16 h and then treated with human recombinant FGF-1 (10 ng/ml), FGF-2 (5 ng/ml), anti-FGF-2 (15 μg/ml), and His-BP1 (6 ng/ml), unless indicated otherwise. The proliferation rate was evaluated after 48 h by the addition of 10 μl/well WST-1 reagent, as suggested by the manufacturer (Roche Molecular Biochemicals). Rabbit polyclonal anti-FGF-2 was purchased from R&D Systems (Minneapolis, MN).

CAM Assay—The CAM assay was carried out as described previously (36). In brief, 2-day-old fertilized chicken eggs were broken open into 35 × 10-mm Petri dishes and incubated at 37 °C for 48 h. Sterile Whatmann filter disks (8 mm in diameter) were prewetted in TBS solution and placed peripherally on the CAM of viable embryos, in between adjacent visible blood vessels. FGF-2 and His-BP1 were placed on the disks as indicated. CAMs were photographed using a digital camera at 0, 12, 24, and 36 h after disk placement. The degree of angiogenesis around each disk was measured using a score from 1 (minimal angiogenesis) to 4 (maximal angiogenesis, with directional growth of new vasculature toward the disk). Scoring was carried out blinded, and the results were averaged. Baseline (no treatment) was subtracted from the average score as indicated.

Statistics and Data Analysis—Graphpad/Prism Software (San Diego, CA) was used for statistical analysis as well as curve fitting to obtain half-maximal inhibitory concentrations (IC$_{50}$) from binding studies. From this, K_d values for FGF-2 were derived by using the Cheng-Prusoff equation ($K_d = IC_{50}/(1 + L/K_d)$), (37) where L is the concentration of the FGF-2 radioligand, and IC$_{50}$ is the half-maximal inhibitory concentration calculated from the competition isotherm of the unlabeled FGF-2.

RESULTS

Generation of Recombinant FGF-BP1 Protein—In previous studies, we investigated the effects of FGF-BP1 by overexpression experiments in FGF-BP1-negative cells or by depletion of endogenous FGF-BP1 from cells using ribozyme targeting (26, 27, 29). Because FGF-BP1 is secreted from cells and acts as an extracellular chaperone, we sought to examine the biological effects of a human recombinant FGF-BP1 when added to the extracellular milieu. For this purpose, two recombinant human FGF-BP1 proteins were produced *in vitro*. A bilaterally hexahistidine-tagged FGF-BP1 protein (His-BP1) was purified from Sf-9 insect cells infected with a baculovirus construct containing nucleotides 197–799 of human FGF-BP1 cDNA (GenBank™ accession number M60047). This protein was used for the functional assays, and its purification is shown in Fig. 1. In addition a glutathione *S*-transferase (GST)-tagged FGF-BP1 designated GST-BP1 was generated in BL21 bacteria using a pGEX-2TK construct that contains the same nucleotides (data

not shown). This protein was used to generate antibodies and used in some of the initial protein-protein interaction studies (see below). To determine whether the protein generated in the Sf-9 cells did indeed represent FGF-BP1, serial elutions obtained from the final affinity chromatography were separated by SDS-PAGE, and a single protein was detected by silver staining and Western blotting with anti-BP1 as well as anti-histidine tag antibodies (Fig. 1, *A–C*). The BP1 protein ran at 34 kDa apparent molecular mass, and the silver staining of a pooled sample shows that the purity of the preparation is >90% (Fig. 1A). The electrophoretic mobility of the His-BP1 protein is slower than that predicted by its molecular mass (26.9 kDa), most likely due to its basic nature, a feature also reported for the bovine BP1 protein (see Ref. 38).

FGF Binding to FGF-BP1 in Cell-free Assays—We first asked whether the recombinant His-BP1 will specifically recognize FGF-2 when the FGF-2 ligand is present at low abundance in a diverse mixture of molecules comprising fetal calf serum. To address this question, we used surface-enhanced laser desorption/ionization protein chip technology coupled with mass spectrometry (34) (see Fig. 1D). This approach was previously applied by us to characterize ligand-receptor interaction when studying pleiotrophin and its receptor, anaplastic lymphoma kinase (35). In the present experimental series, the His-BP1 protein was immobilized on a protein chip and incubated with FGF-2 that had been mixed with growth media containing 10% fetal calf serum. FGF-2 comprised only a very small portion of the overall preparation used as the input (*arrowhead* in Fig. 1D, *1, input*). However, FGF-2 was specifically recognized by the immobilized His-BP1 in this mixture (*arrowhead* in Fig. 1D, *2*), and only nonspecific binding was observed without the immobilized His-BP1 (Fig. 1D, *3*). From this, we conclude that FGF-2 does indeed bind specifically to His-BP1, even when the growth factor is only present at very low abundance in a complex mixture of proteins and other molecules, and that no other ligand for His-BP1 is present in fetal calf serum.

We next sought to quantitate FGF binding to the His-BP1 recombinant protein. For this, a cell-free 96-well binding assay was established using immobilized His-BP1 as a bait and ^{125}I-FGF-2 as a ligand. As shown in Fig. 2A, His-BP1 (*bottom panel*) and GST-BP1 (*top panel*) bound to the radiolabeled FGF-2 in a dose-dependent manner. The bacterial GST-BP1 displayed less specific and more nonspecific binding to ^{125}I-FGF-2 per unit of protein in comparison with the eukaryotic His-BP1, and we thus decided to use the latter as the major tool for our additional studies. To support the specificity of His-BP1 binding to the FGF-2 radioligand, competition assays were performed with excess cold FGF-2, FGF-1, or His-BP1 as well as epider-

FIG. 2. **Binding of FGF-2 to His-BP1 and competition by different agents.** *A*, binding of different concentrations of ^{125}I-FGF-2 to GST-BP1 (*top panel*) and His-BP1 (*bottom panel*) immobilized in 96-well plates. *B–G*, competition of His-BP1, FGF-1, FGF-2, heparin, heparan sulfate, and pentosanpolysulfate for ^{125}I-FGF-2 binding to immobilized His-BP1. ^{125}I-FGF-2 binding obtained from wells containing immobilized His-BP1 (●; 100%) in comparison to blocking solution only (○; 0%) is shown. The data are representative of at least four independent experiments, in which each sample was run in triplicate.
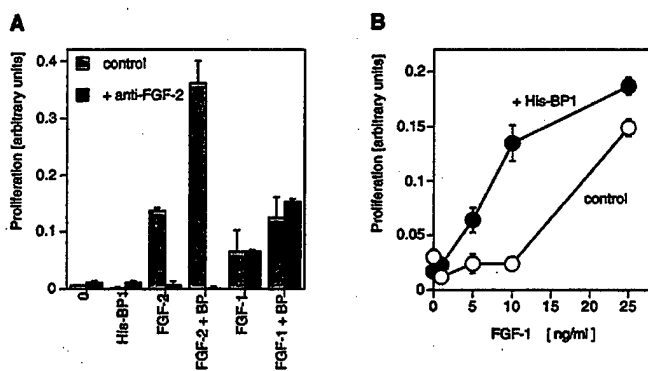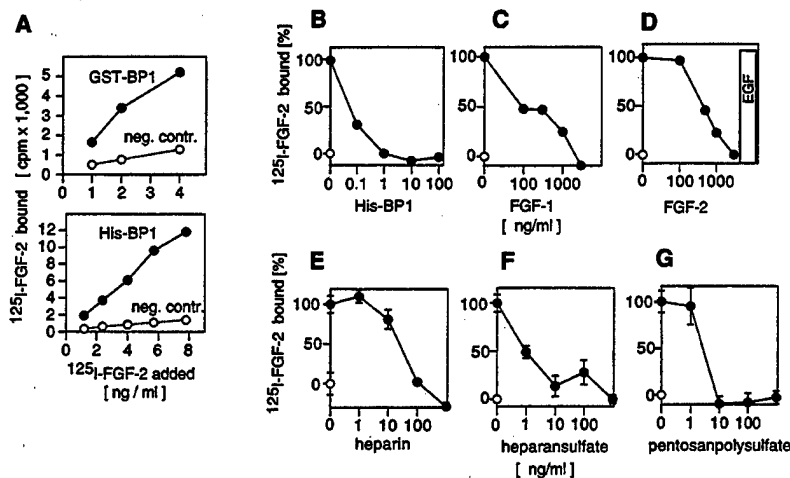
FIG. 3. **Effect of His-BP1 on FGF-1- and FGF-2-mediated mitogenesis in NIH-3T3 fibroblasts.** *A*, cells were treated for 48 h with FGF-1 (10 ng/ml) or FGF-2 (5 ng/ml) ± His-BP1 (6 ng/ml) in the absence (▨) or presence (■) of anti-FGF-2 antibody. *B*, concentration-response curve of FGF-1 in the absence (○) or presence of His-BP1 (●). The proliferation rate was measured as described under "Materials and Methods," and the data shown are representative of three independent experiments.
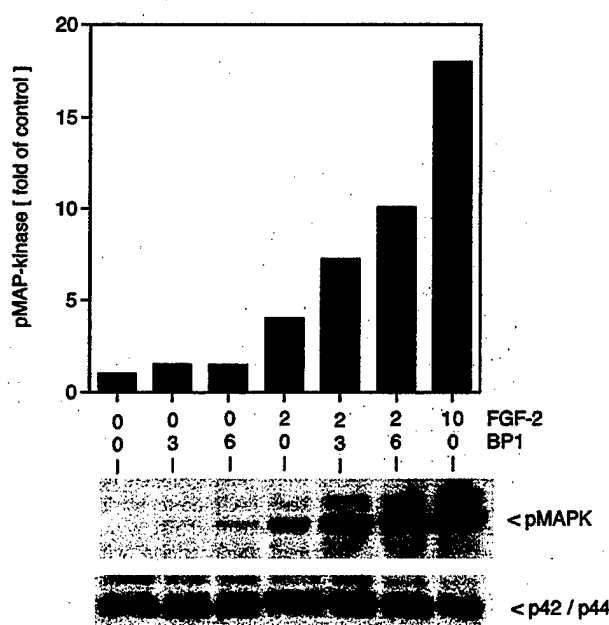
FIG. 4. **Effect of His-BP1 on FGF-2-induced ERK2 activation in NIH-3T3 fibroblasts.** Cells were starved overnight and treated for 5 min with different concentrations of FGF-2 and/or His-BP1, as indicated (ng/ml). Controls were left untreated. 50 μg of total cell lysates were separated by 10% SDS-PAGE, transferred onto polyvinylidene difluoride membranes, and immunoblotted with anti-phosphotyrosine monoclonal antibody (*pMAPK*). Detection of endogenous ERK2 in the protein extracts was determined by Western blot analysis, using anti-pan ERK monoclonal antibodies (*p42/44*). Quantitation of bands was obtained by densitometry and is expressed relative to control (*bar graph*).

mal growth factor as a nonspecific growth factor control. As shown in Fig. 2*B*, increasing concentrations of His-BP1 were able to inhibit the binding of ^{125}I-FGF-2 to the immobilized His-BP1, and an ~10-fold excess of the His-BP1 in solution completely inhibited FGF-2 binding. FGF-2 also competed for FGF-2 radioligand binding (Fig. 2*D*). From a series of such competition assays, we calculated an apparent dissociation constant (K_d) value of ~10 nM for FGF-2 binding to His-BP1 (for details, see "Materials and Methods"). Furthermore, in support of the original report on FGF-BP1 by Wu *et al.* (28), we found that FGF-1 also competed with FGF-2 for its binding to His-BP1 (Fig. 2*C*). Epidermal growth factor was used as a negative control and did not did not inhibit FGF-2 binding to the immobilized His-BP1 even at 10 μg/ml (*bar* in Fig. 2*D*), supporting a specific interaction of FGF-BP1 and FGF-2.

Earlier studies from our laboratory have shown that FGF-BP1 lowers the affinity of heparin for FGF-2 (29) in support of the notion that FGF-BP can release FGF-2 from its local storage on glycosaminoglycans in the extracellular matrix (27). Conversely, based on this mechanism of action of FGF-BP1, we speculated that glycosaminoglycans in solutions should be able to disrupt binding of FGF-2 to FGF-BP. We thus studied the effects of heparin, heparan sulfate, and pentosanpolysulfate on the binding of FGF-2 to His-BP1 (Fig. 2, *E–G*). The extracellular matrix glycosaminoglycan heparan sulfate inhibited binding of FGF-2 to the immobilized His-BP1 at very low concentrations (IC$_{50}$ = 1 ng/ml), and the semisynthetic heparinoid

pentosanpolysulfate (39–41) and the anticoagulant heparin were somewhat less potent (IC$_{50}$ = 3 and 30 ng/ml). All of these heparinoids completely inhibited binding of FGF-2 to the immobilized His-BP1, and we propose from these data that the heparin-binding domain in FGF-2 overlaps with the binding domain that interacts with FGF-BP. Also, this mutually exclusive binding of FGF-2 to FGF-BP or to a glycosaminoglycan supports the notion of FGF-BP as an extracellular chaperone molecule that releases locally stored FGFs (26, 27, 30, 42).

FGF-BP1 Enhances FGF-induced Proliferation in NIH-3T3 Fibroblasts—After we demonstrated the ability of the recombinant human FGF-BP1 to specifically bind FGF-1 and -2 in a cell-free system, we sought to determine the effect of this interaction on biological activity *in vitro*. FGF-1 and -2 are potent mitogens for a number of cell lines, including fibroblasts and

FIG. 5. **Effect of His-BP1 on FGF-2-induced angiogenesis in the CAM assay.** Filter disks with FGF-2 (100 ng) ± His-BP1 (as indicated) were placed on CAMs of 4-day-old chicken embryos. *A* and *B*, photograph of angiogenesis on the CAMs scored as 1 (*A*) and 3 (*B*) at 24 h. *Bar*, 1 mm. *C*, quantitation of the assays; $p = 0.015$ is the overall p value from analysis of variance. *, $p < 0.05$; **, $p < 0.01$ (comparison relative to the respective control sample).
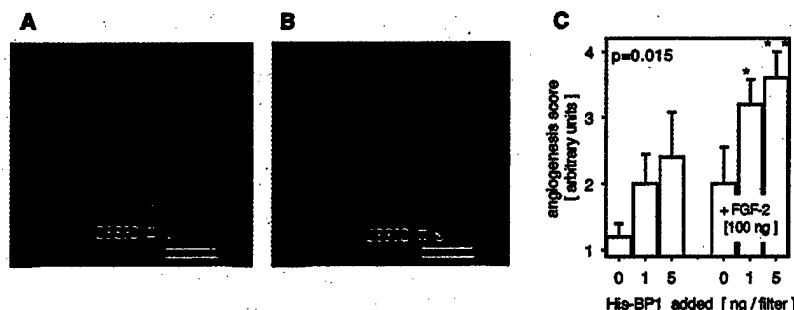
endothelial cells (17), and we used NIH-3T3 fibroblasts as an experimental model system to study the effect of His-BP1 on FGF-induced proliferation (Fig. 3). To control the effectiveness of FGF-2 mitogenic action, we first assessed the ability of the growth factor to induce cell proliferation. NIH-3T3 cells were serum-deprived overnight and grown for 48 h in the presence or absence of different concentrations of FGF-2 to establish a dose-response curve (data not shown). We then stimulated the cells with a submaximally effective concentration of FGF-2 (5 ng/ml) and added His-BP1 (6 ng/ml) (Fig. 3A). Whereas no proliferation was detected when cells were grown in the presence of His-BP1 alone, the addition of FGF-2 enhanced the mitogenic activity of the growth factor. To rule out the possibility that interaction of His-BP1 with a mitogenic factor other than FGF-2 was responsible for the His-BP1-mediated effect, we included an FGF-2-specific antibody. The anti-FGF-2 antibody blocked the FGF-2-dependent cell growth and completely inhibited the synergistic effect of His-BP1 and FGF-2. As a control, the anti-FGF-2 antibody failed to reduce FGF-1-dependent cell growth as well as the synergistic effects between His-BP1 and FGF-1. As for FGF-2, a concentration-response curve for FGF-1 showed that His-BP1 enhances the effects of low concentrations of FGF-1 ($p < 0.01$; analysis of variance) and does not increase the maximal effect of the growth factor (Fig. 3B). Taken together, these results establish that the synergistic interaction between His-BP1 and FGF-1- or FGF-2-stimulated NIH-3T3 mitogenesis is specific and dependent on the presence of the respective growth factor.

FGF-BP1 Positively Modulates FGF-2-induced ERK2 Activation in a Dose-dependent Fashion—ERK2 plays an important role in transducing proliferative signals from receptor tyrosine kinases (18). In particular, engagement of FGFRs by their extracellular ligands, such as FGF-1 and FGF-2, has been extensively reported to induce activation of the Ras/ERK cascade (16, 43). Consistent with these observations and in light of our results, we next decided to examine the early signaling events elicited by the specific interaction between FGF-2 and His-BP1 (Fig. 4). Interestingly, we found that FGF-2-dependent ERK2 activation, as determined by immunoblot analysis with an anti-phosphotyrosine antibody, was significantly enhanced in NIH-3T3 fibroblasts when cells were co-stimulated for 5 min with different concentrations of FGF-2 and His-BP1. In particular, 3 and 6 ng/ml His-BP added to FGF-2 (2 ng/ml) exhibited an enhancement of phosphorylation of 1.8- and 2.5-fold, respectively, when compared with the levels obtained with FGF-2 alone (Fig. 4, *bar graph*). The same results were obtained in immunoprecipitation studies (data not shown). In addition, as shown in the *bottom panel* of Fig. 4, the levels of expression of ERK2 were not affected by these treatments and, consistent with the phospho-MAPK blots, a mobility shift of the lower ERK2 band due to phosphorylation was obvious. From these findings, we conclude that FGF-2-dependent phosphorylation of ERK2 is synergistically modulated by His-BP1.

FGF-BP1 Enhances FGF-2-dependent Angiogenesis in

Vivo—FGF-2 has been shown to be a powerful inducer of angiogenesis both *in vitro* and *in vivo* (2, 21, 44). As an experimental approach to investigate the effects of FGF-BP1 on FGF-2-mediated angiogenesis *in vivo*, we used the chick embryo CAM assay. As shown in Fig. 5, FGF-2 and His-BP1 induce an angiogenic response on their own. The baseline effect of FGF-BP1 on its own is likely due to locally stored FGFs from the chicken embryo. However, simultaneous stimulation with both FGF-2 and FGF-BP1 resulted in a significant enhancement of this response. These findings support the notion that FGF-BP1, by its cooperative interaction with FGF-2, is a positive regulator of FGF-2-mediated angiogenesis *in vivo*.

DISCUSSION

FGF-BP1 is a secreted protein that binds FGF-2 and is hypothesized to mobilize FGF-2 from its storage in the ECM (28). Previous studies have found that endogenous FGF-BP1 is overexpressed in several cancers (26, 27, 30). Additionally, depletion of FGF-BP1 mRNA has been shown to abrogate the angiogenesis-dependent growth of ME-180 squamous cell carcinoma and Ls174T colon cancer cells when implanted in athymic nude mice (27). Because endogenous FGF-BP1 plays a critical role in tumor growth and angiogenesis, we set out to explore the effects of exogenously added FGF-BP1 protein on FGF-dependent cellular responses.

For our experiments, we produced and purified human recombinant, polyhistidine-tagged FGF-BP1. This His-BP1 protein gave a single band after SDS-PAGE by silver staining as well as by immunoblotting, and recombinant His-BP1 binds radiolabeled FGF-2 *in vitro*. This binding is specific because it is competed by excess concentrations of cold FGF-1 and -2 or recombinant FGF-BP1 and not by an unrelated growth factor, epidermal growth factor. Heparinoids inhibit binding between FGF-2 and FGF-BP1 at low concentrations (nanograms/milliliter), and we conclude from this that binding of FGF-2 to heparinoids and to FGF-BP1 is mutually exclusive. This lends additional support to the role of FGF-BP1 as a chaperone that can shuttle FGFs from their glycosaminoglycan storage. The His-BP1 purified as a monomeric protein and chemical cross-linking studies of radiolabeled FGF-2 with His-BP1 suggested that these proteins bind at a 1:1 ratio.[2]

There have been conflicting reports with regard to whether FGF-BP1 binds FGF-1. In the original description of FGF-BP1, Wu *et al.* (28) found that FGF-BP1 purified from the media of A431 human epidermoid carcinoma cell cultures was also able to bind FGF-1. In contrast, Lametsch *et al.* (38) reported that FGF-BP1 purified from bovine prepartum mammary secretion binds FGF-2, but not FGF-1. Nevertheless, in our experiments, FGF-1 completely inhibited FGF-2 binding to the human recombinant FGF-BP1, thus demonstrating an interaction of FGF-1 with FGF-BP1. Furthermore, FGF-BP1 synergized with FGF-1 as well as with FGF-2 in the proliferation assays with

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[2] A. Al-Attar and A. Wellstein, unpublished data.

NIH-3T3 cells (Fig. 3), and the effect of FGF-BP1 on both of these FGFs was indistinguishable.

The MAPK pathway has been studied extensively in the stimulation of quiescent cells with mitogenic factors and is generally considered to be responsible for the initiation of cellular growth. Of note, in different cellular systems, it has been shown that the specific interaction of FGF-2 with its receptor and the subsequent FGFR activation trigger a downstream signal cascade that culminates with the activation and phosphorylation of ERK2 (16). To investigate the role of recombinant exogenous FGF-BP1 on FGF-2-mediated early intracellular biological responses, phosphoprotein analysis was performed in NIH-3T3 murine fibroblasts. Consistent with previous findings that demonstrated the physical interaction of FGF-BP1 with FGF-2 (28), we now provide evidence that FGF-BP1 exerts positive and synergistic modulation of FGF-2-mediated signaling by enhancing the growth factor-dependent ERK2 phosphorylation. Interestingly, we show that at concentrations of FGF-2 not sufficient to elicit maximal ERK2 activation, FGF-BP1 significantly amplifies the FGF-2-mediated response in a dose-dependent manner. Sustained activation of the ERK2 signal transduction pathway often controls the stimulation of cell proliferation (45, 46), and ERK2 activation is required for proliferation of fibroblasts *in vitro* (43). In addition to ERK2, FGF-2-dependent mitogenic signal transduction pathways lead to the activation of phospholipase Cγ and p70S6K, respectively. However, it has been observed that phospholipase Cγ activation is likely not to be responsible for FGF-2-mediated NIH-3T3 mitogenesis, nor is the signal that emanates from p70S6K sufficient to induce cell proliferation (47–49). Indeed, coincident with the ERK2 activation, we found that the interaction of FGF-BP1 with FGF-2 also elicits a dramatic enhancement of FGF-2-mediated NIH-3T3 proliferation. This supports the notion of FGF-BP1 as a chaperone molecule that will serve as a positive modulator of FGF-2-dependent growth controlled by the ERK2 pathway.

FGF-2 is a potent angiogenic molecule, and previous studies have shown that it can induce neovascularization in the chicken embryo CAM assay. Here, we use this experimental model to address whether recombinant FGF-BP1 can act synergistically with FGF-2 to cause new and directed blood vessel growth. We found that the angiogenic response seen with the addition of both recombinant FGF-BP1 and FGF-2 was significantly greater than that seen with the addition of FGF-2 alone. Furthermore, FGF-BP1 treatment led to a more rapid establishment of directed blood vessel growth when added to FGF-2 (see Fig. 5*A*, *left versus right panel*). Interestingly, we found that FGF-BP1 added to the CAM, without exogenous FGF-2, was able to induce angiogenesis in a dose-dependent manner on its own; we speculate that this is due to endogenous FGFs present in the CAM. This finding is also consistent with an earlier report in which transfection of FGF-BP1 into a human adrenocortical carcinoma cell line (SW-13) induced the growth of highly vascularized tumors in athymic nude mice (26), and reduction of FGF-BP1 message in ME-180 cells reduced their angiogenic stimulus during tumor growth (27). In addition, all-*trans*-retinoic acid has been shown to down-regulate FGF-BP1 mRNA levels in tumors grown from the ME-180 cells and, coincident with that, the extent of tumor angiogenesis (50).

Binding proteins have been described for other cytokines, and the most relevant two binding protein families are those for insulin-like growth factor and transforming growth factor β. Latent transforming growth factor β-binding proteins and insulin-like growth factor-binding proteins have been shown to bind and protect their respective ligands from degradation and can positively or negatively modulate their ligands' functional activities (51, 52). Each of these binding proteins represents a family of multiple proteins, with homologous members found in different tissues and species. Similarly, human FGF-BP1 has homologues in chicken, zebrafish, cow, mouse, and rat (2, 28, 29, 38). Recently, we found a novel FGF-BP, designated FGF-BP2, that is also located on chromosome 4p16, in close proximity to FGF-BP1. The amino acid sequences of these two proteins contain eight cysteine residues that are conserved across different species and between the FGF-BP genes. This suggests identical disulfide bond formation and similar tertiary structure. It will be interesting to see to what extent the different FGF-BPs contribute to the diversity of activities by the more than 20 FGFs (2).

In conclusion, our studies suggest that FGF-BP1 represents an important regulatory factor that positively modulates FGF-mediated cellular responses, such as signaling, proliferation, and angiogenesis.

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